Cancer Immunol Immunother (2014) 63:45–57
DOI 10.1007/s00262-013-1480-0

FOCUSSED RESEARCH REVIEW

Clinical evaluation of systemic and local immune responses in cancer: time for integration

Dmitriy W. Gutkin · Michael R. Shurin

Received: 12 August 2013 / Accepted: 23 September 2013 / Published online: 8 October 2013

Abstract The immune system has a dual role in cancer development and progression. On the one hand, it can eradicate emerging malignant cells, but on the other hand, it can actively promote growth of malignant cells, their invasive capacities and their ability to metastasize. Immune cells with predominantly anti-tumor functionality include cells of the innate immune system, such as natural killer cells, and cells of adaptive immunity, such as conventional dendritic cells and cytotoxic T lymphocytes. Immune cells with predominantly pro-tumor functionality include a broad spectrum of cells of the innate and adaptive immune system, such as type 2 neutrophils and macrophages, plasmacytoid DC, myeloid-derived suppressor cells and regulatory T lymphocytes. The presence of immune cells with tumor-suppressive and tumor-promoting activity in the cancer microenvironment and in peripheral blood is usually associated with good clinical outcomes and poor clinical outcomes, respectively. Significant advances in experimental and clinical oncoimmunology achieved in the last decade open an opportunity for the use of modern morphologic, flow cytometric and functional tests in clinical practice. In this review, we describe an integrated approach to clinical evaluation of the immune status of cancer patients for diagnostic purposes, prognostic/predictive purposes (evaluation of patient prognosis and response to treatment) and for therapeutic purposes.

Keywords Tumor immunoenvironment · Cancer · Immunomonitoring · Tumor-infiltrating leukocytes · CITIM 2013

Introduction

It is now well accepted that the immune system has a dual role in cancer development and progression. It can eradicate malignant cells by an orchestrated action of innate and adaptive branches, thus preventing tumor growth. On the other hand, it can actively promote growth of malignant cells, their invasive capacities and ability to metastasize. This controversial role of the immune system is described in the concepts of immune surveillance and immune editing. The specific role of several key immune cells and cytokines has been elucidated in numerous in vitro studies, animal experiments and clinical trials. Here, we will describe the main types of immune cells with tumoricidal and pro-tumorous activities focusing on the practical significance of their evaluation in cancer patients for diagnostic (immune status of cancer patients), prognostic/predictive (prognosis and response to treatment) and therapeutic purposes.

We are now at the point when the broad knowledge accrued by experimental immunology is entering clinical practice. Laboratory techniques designed to evaluate numbers, phenotype and functionality of immune cells are becoming commonly available (Tables 1, 2), and soon the assessment of immune status will become a part of a

This paper is a Focussed Research Review based on a presentation given at the Third International Conference on Cancer Immunotherapy and Immunomonitoring (CITIM 2013), held in Krakow, Poland, on 22–25 of April 2013. It is part of a CII series of Focussed Research Reviews and meeting report.

D. W. Gutkin (✉) · M. R. Shurin
Departments of Pathology, University of Pittsburgh, Pittsburgh, PA, USA
e-mail: dwgutkin@hotmail.com

M. R. Shurin
Departments of Immunology, University of Pittsburgh, Pittsburgh, PA, USA

Table 1 Commonly used immunohistochemical stains for tumor-infiltrating leukocytes

Target	Staining	Properties
B lymphocytes	CD20	33 kD protein, pan-B lymphocyte marker
T lymphocytes	CD3	Part of T cell receptor complex
T helper cells	CD4	Cell-surface glycoprotein, co-receptor for the T Cell receptor complex
Cytotoxic T cells	CD8	Cell-surface glycoprotein, co-receptor for the T cell receptor complex
Regulatory T cells	CD25	IL-2R α chain, expressed by early progenitors of the T and B lineage as well as by activated mature T and B lymphocytes
	FoxP3	Member of the fork head/winged-helix family of transcriptional regulators, in CD25+ CD4+ regulatory T cells
Natural killer cells	CD56	Glycosylated transmembrane protein, expressed by NK cells, a subset of T cells, and neuroectodermal-derived cells
	CD57	Human natural killer-1, expressed on NK cells
Neutrophils	Myeloperoxidase	Enzyme in the granules of neutrophils and to a lesser extent the granules of monocytes
	CD15	Cell-surface membrane protein, expressed on neutrophils, a subset of tissue macrophages and activated T lymphocytes
	CD66b	Member of the immunoglobulin superfamily, expressed on neutrophils
Macrophages	CD68	Glycoprotein of cytoplasmic granules
	HLA-DR	Major histocompatibility complex, class II, cell-surface receptor, marker of M1 activation
	CD163	Transmembrane protein, marker of M2 activation
	CD204	Macrophage scavenger receptor 1, marker of M2 activation
Immature myeloid DCs	CD1a	49 kDa cell-surface glycoprotein expressed in association with beta-2-microglobulin; expressed predominantly in early steps of DC maturation
	CD209/DC-SIGN	DC-specific adhesion receptor that mediates DC binding to ICAM-3; presumably mediates the recognition of non-self and the presentation of foreign antigens; can regulate important adhesion processes
	CD207/Langerin	C-type lectin responsible for the formation of Birbeck granules, a typical hallmark for DCs of Langerhans type
Mature myeloid DCs	CD83	40-45 kDa glycoprotein expressed predominantly in the late steps of DC maturation; CD83+ DCs co-express the highest levels of HLA II
	CD86	Membrane protein of the immunoglobulin superfamily, which provides a co-stimulatory signal necessary for T cell activation and survival
	CD208/DC-LAMP	Member of the lysosomal-associated membrane protein (LAMP) family; plays an important role in antigen processing and MHC-II restricted antigen presentation
Plasmacytoid DCs	CD123	IL-3 receptor α-chain involved in cell signaling for cell growth and differentiation

routine evaluation of cancer patients, with the potential to significantly improve the overall clinical outcome.

Immune cells with the anti-tumor functions

Immune cells with predominantly anti-tumor functionality include cells of both the innate and adaptive immune system, such as natural killer (NK) cells, conventional dendritic cells and cytotoxic T lymphocytes [1]. The cells of the innate immune system are the first to detect the emergence of neoplastic cells. Numerous studies showed that the presence of immune cells with the potent anti-tumor function in the tumor microenvironment is associated with good clinical outcome, suggesting the importance of their assessment for clinical purposes.

Natural killer cells

NK cells play a major role in the elimination of tumor cells that have lost MHC expression [2, 3].

In general, NK cell density is low in human neoplasms, with the exception of some renal cell carcinomas [4–7]. In regard to the correlation of NK cell density at the tumor mass with the clinical course and prognosis, the majority of studies showed favorable prognostic value, specifically, for gastric carcinoma [5], colorectal carcinoma [6] and pulmonary adeno- and squamous cell carcinoma (SCC) [4, 8]. Confirming the importance of a spatial distribution of NK cells in tumor tissue, Al-Shibli et al. [8] showed that high density of stromal NK cells was an independent positive prognostic factor for disease-specific survival in pulmonary carcinoma, whereas high density of NK cells within tumor

Table 2 Commonly used flow cytometry markers for immunostimulatory and immunosuppressive leukocytes

Target	Flow cytometry pattern
B lymphocytes	CD20+, CD19+
T lymphocytes	CD2+, CD3+,
T helper cells	CD2+, CD3+, CD4+
Cytotoxic T cells	CD2+, CD3+, CD8+
Regulatory T cells	CD2+, CD3+, CD25+, FoxP3+
Natural killer cells	CD3−, CD56+, CD 57+
Neutrophils	CD13+, CD15+, CD33+
Macrophages	CD68+, HLA-DR+, CD163+, CD204+
Immature myeloid DCs	CD1a, CD 209+, CD 207+
Mature Myeloid DCs	CD 83+, CD86+, CD208+
Plasmacytoid DCs	CD 123+
MDSCs granulocytic	CD15+, CD66b+, CD33+
MDSCs monocytic	CD14+

islets was not. On the other hand, in soft tissue sarcomas, there was no correlation between NK cell density in tumors or peritumoral tissues and the patients' prognosis [9].

Conventional dendritic cells

Density of DCs in the tumor mass varies depending on the type of malignancy. For instance, in breast carcinoma, tumor-infiltrating DCs are detected in 30–50 % of tumors [10]. In two main types of pulmonary non-small cell carcinoma (adenocarcinoma and SCC), DCs are found in 60–80 % of tumors [11, 12]. However, DC density in two types of pulmonary neuroendocrine tumors (small cell carcinoma and carcinoid tumor) is usually very low [13]. Katsenelson et al. found different populations of DCs, including CD1a+ immature DCs (iDCs) and CD83+ mature DCs (mDCs), in small cell carcinoma, but samples of carcinoid tumor were devoid of DCs [14]. In transitional cell carcinoma of the urinary bladder, a dense infiltrate of S100+ DCs is detected in 50 % of cases [15]. In oral SCC, density of DC infiltrates was low in 20 % of specimens, intermediate in 42 % of specimens and high in 37 % of specimens [16]. Pancreatic carcinoma is characterized by a paucity of tumor-infiltrating DCs; significant numbers of S100+ DCs and CD1a+ iDCs were found in only 4 % of tumors [17].

Unlike other tumor-infiltrating leukocytes, the density of tumor-infiltrating DCs may be lower in the tumor than in the corresponding normal tissue. For example, Troy et al. compared the number of DCs in prostate carcinoma and adjacent normal prostatic tissue and found that there were significantly fewer CD1a+ iDCs in prostate cancer compared with normal prostatic tissue and only a small subset of DCs expressed markers of activation, such as CD83 and CD86 [18]. The density of CD83+ mDC is also significantly lower in gastric cancer tissue than in normal gastric tissue [19]. As discussed below, the low density of tumor-infiltrating DCs may present a survival advantage to malignant tumors and thus be a mechanism of immune escape. Vakkila et al. [20] compared DC density in pediatric and adult tumors. While DCs were present in adult tumors (colon carcinoma, breast carcinoma, esophageal carcinoma), tumor-infiltrating DCs were virtually absent in pediatric malignancies (Ewing's sarcoma, rhabdomyosarcoma, hepatoblastoma, neuroblastoma, Wilms' tumor). Inflammatory infiltrate in pediatric tumors was composed mainly of macrophages, whereas in adult tumors, DCs formed 37 % of leukocytes within the tumor islands and 25 % around the tumors. The reason for this striking difference merits further investigation.

When present in the tumor mass, DCs can be seen within cancer nests, in tumor stroma, and in peritumoral areas. Their spatial distribution seems to depend on the type of the tumor. In colorectal carcinoma, infiltration of tumor stroma by DCs was significantly higher than in tumor islets [21]. In contrast, in pulmonary non-small cell carcinoma, DCs were located predominantly in cancer nests and their number correlated with the extent of cancer cell apoptosis. In areas of scattered DC distribution, only a few apoptotic tumor cells can be detected, while in the areas of DC aggregations, apoptotic tumor cells were significantly more abundant [22].

Spatial distribution of tumor-infiltrating DCs seems to depend on the level of their maturation. It was demonstrated that the majority of iDCs are located within the tumor nests, while mDCs are present in the stroma. For example, in breast carcinoma, CD1a+ iDCs were retained predominantly within the tumor epithelium, whereas CD83+ and LAMP+ mDCs were confined to peritumoral areas [23, 24]. Similar data were reported for colonic adenocarcinoma [25], oral SCC [26], biliary carcinoma [27], transitional cell carcinoma of the urinary bladder [28] and melanoma [29].

In regard to the correlation between DC density and tumor grade, the majority of studies showed higher DC density in well-differentiated than in poorly differentiated neoplasms. This correlation was reported in pulmonary non-small cell carcinoma [13], prostate carcinoma [30] and endometrial carcinoma [31]. However, in breast carcinoma, the number of tumor-infiltrating DCs was higher in high-grade tumors [23]. Correlation of DC density with tumor stage was performed by Kikuchi et al. [32] who found that in head and neck cancer, the numbers of iDCs were greater in patients with lower stage of the disease and decreased with tumor progression. Interestingly, mDC density showed the reverse correlation. Significant decrease in iDCs with simultaneous increase in

mDCs was also demonstrated in the progression steps of cervical SCC [33].

Correlations of the density of tumor-infiltrating DCs with clinical outcome were extensively studied for numerous types of tumors. In the majority of them, high density of tumor-infiltrating DCs (especially mDCs) was a favorable prognostic feature. In fact, some of the studies found the density of tumor-infiltrating mDCs to be a better predictor of clinical outcome than other well-established parameters [34]. In a large cohort of patients with pulmonary non-small cell carcinoma, increasing density of stromal DCs was associated with increased disease-specific survival (DSS) [8, 35]. In breast carcinoma, high mDC density was also a favorable prognostic marker. At the same time, no correlation was found for total DC and iDC density [36, 37]. The same correlations were found in colonic carcinoma [21], gastric carcinoma [19], hepatocellular carcinoma [38], biliary carcinoma [27], oral SCC [16] and melanoma [39].

T lymphocytes

High density of tumor-infiltrating CD3+ T cells has been associated with favorable prognosis in various types of cancers. It was reported for pulmonary non-small cell carcinoma [40], colorectal carcinoma [41], gastric carcinoma [42] and ovarian carcinoma [43]. Recently, Gooden et al. [44] performed a meta-analysis of 33 large clinical studies and found a strong positive effect of CD3+ tumor-infiltrating lymphocytes on patients' survival.

Among specific subtypes of tumor-infiltrating lymphocytes, cytotoxic T cells have also been associated with better survival in many types of cancer, including pulmonary non-small cell carcinoma [8, 35], colorectal carcinoma [45], esophageal carcinoma [46], urothelial carcinoma [47], cholangiocellular carcinoma [48], endometrial carcinoma [49] and ovarian cancer [50]. However, in other studies, CD8+ T cell density was not found to correlate with prognosis in pulmonary non-small cell carcinoma [51], esophageal SCC [52] and soft tissue sarcoma [53]. In a meta-analysis, CD8+ T cells had a positive effect of on patients' survival [44].

Tumor-infiltrating T helper cells are not studied as extensively as CTLs; however, several reports indicate their favorable prognostic significance. Remarkably, this effect depends on a spatial distribution of the cells. In a study of the prognostic role of epithelial and stromal CD4+ T cells in patients with resected non-small cell carcinoma, Al-Shibli et al. [54] found that increasing numbers of CD4+ in tumor stroma, but not in cancer islets, correlated significantly with improved DSS. Other groups reported similar results [51, 55]. High density of CD4+ T cells also correlated significantly with an improved survival in patients with soft tissue sarcoma [56]. In addition to the reports on the individual role of T cell types, several studies found a favorable prognostic effect of concurrent infiltration by CD8+ cells and CD4+ cells. Specifically, this effect was shown in pulmonary non-small cell carcinoma [57] and esophageal SCC [58].

Immune cells with pro-tumor functions

Immune cells with predominantly pro-tumor functionality include a broad spectrum of cells of the innate and adaptive immune system, such as type 2 neutrophils, type 2 macrophages, plasmacytoid DCs, myeloid-derived suppressor cells (MDSCs) and regulatory T (Treg) lymphocytes. Their presence in the tumor microenvironment and peripheral blood is associated with a poor clinical outcome.

Neutrophils

Neutrophils represent the main population of leukocytes in the blood and are considered to be the first line of immune response to tissue injury. Neutrophils make up a significant portion of the inflammatory cell infiltrate found in a wide variety of human cancers [59–62]. Although neutrophils are well equipped to kill malignant cells by several mechanisms, in the tumor microenvironment they tend to have the opposite effect and directly induce tumor cell proliferation through the expression of growth-promoting bioactive molecules. Specifically, neutrophil-derived hepatocyte growth factor has been correlated with increased tumor growth in lung cancers [61].

Even more important for tumor development are the effects of neutrophils infiltrating central tumor stroma and the peritumoral invasive margin. These cells promote tumor progression through remodeling of the extracellular matrix, enhancing tumor cell migration, and invasion and modulating angiogenesis [63–65].

The majority of the clinical studies regarding tumor-infiltrating neutrophils have demonstrated that their presence and high density are associated with poor clinical outcomes, including decreased survival. This correlation has been shown for pulmonary adenocarcinoma [62], gastric adenocarcinoma [66], colorectal carcinoma [67] and renal cell carcinoma [60]. For example, the presence of intratumoral neutrophils decreased the 5-year recurrence-free survival rate from 87 to just 53 % [60]. However, in some studies, tumor-infiltrating neutrophils were not found to be associated with cancer prognosis [59, 68] or were associated with reduced mortality risk [69].

Macrophages

Macrophages are increased in tumors compared with healthy tissues [70] and constitute a major component of the leukocyte infiltrate in many malignant tumors [71]. However, their density varies widely even in tumors of the same origin.

Macrophages are polarized into two functionally distinct types M1 and M2, most likely under the influence of tumor-derived factors (e.g., TGF-β). M1 macrophages produce high levels of IL-12, IL-23, TNF-α, IL-1, IL-6, CXCL10, iNOS and effector molecules, such as reactive oxygen and nitrogen intermediates and TNF-α, and thus may display potent anti-tumor effects. M2 macrophages express high levels of IL-10, IL-1R antagonist, CCL22, scavenger receptors, arginase I and CD163 [72]. M2 macrophages promote tumor growth and metastasis by secreting MMP-9, angiogenic factors and immunosuppressive cytokines [35, 73–76]. M1 macrophages are located predominantly in tumor islets, whereas M2 macrophages are present predominantly in tumor stroma. Unfavorable prognostic role of M2 macrophages was demonstrated in pulmonary [77], pancreatic [78], renal cell [79] and endometrioid [80] carcinomas.

Plasmacytoid DCs

While present in the tissues at low numbers in a steady state, pDCs accumulate in lymphoid and non-lymphoid tissues under different pathological conditions [81]. They commonly represent a minor fraction (10–15 %) of the infiltrating immune cells [82], but at least in some tumors they were found to be the most abundant DC subset [83].

Accumulation of pDCs in tumors has been directly demonstrated in primary carcinomas of different organs (breast, ovary, head and neck, lung, skin, cervix, prostate and liver), as well as, cutaneous melanoma [83–85]. Numerous experimental and clinical evidence shows that pDCs possess immunosuppressive and tolerogenic properties and promote tumor growth and progression. Tumor-infiltrating pDCs are defective in IFN production and secrete immunosuppressive soluble factors responsible for tumor progression [83, 85]. Tumor-infiltrating pDCs express IDO and secret Granzyme B, which are involved in inhibition of T cell activation and immunosuppression [86, 87]. In addition, pDCs can drive CD4+ T cell polarizations to CD4+ CD25+ Foxp3+ Treg cells, leading to anergy and immune suppression and favoring the immune escape [88].

These findings have strong clinical correlations: prognosis of different types of tumors is inversely related to the density of tumor-infiltrating pDCs. Negative prognostic influence of pDCs has been demonstrated in ovarian cancer [83], breast carcinoma [36] and oral SCC [26]. For instance, CD123+ pDC infiltration was found in 13 % of the breast carcinoma and their presence was strongly associated with shorter overall survival and relapse-free survival and was found to be an independent adverse prognostic factor [36].

Regulatory T cells

Increased levels of CD4+ CD25+ Tregs have been reported in peripheral blood and the tumor microenvironment of patients with non–small cell lung carcinoma [89], gastrointestinal malignancies [90], ovarian cancer [91], SCC of the head and neck [92], hepatocellular carcinoma [93], breast cancer, pancreatic cancer [94] and prostate carcinoma [95]. Tregs variably present within the tumor microenvironment [96]. They usually represent a small fraction of tumor-infiltrating lymphocytes (5–10 % of CD4+ cells), but may have a significant influence on tumor development [97, 98]. It has been shown that the amount of Treg cells is higher in tumors than in normal tissues due to an active recruitment of these cells into the tumor bed [99]. Accumulation of Tregs may be associated with disease progression [100, 101]. High percentage of Treg cells in various neoplasms creates the immune suppressive microenvironment that curbs anti-tumor immunity, thus promoting tumor growth [98].

Not only the number of Tregs, but also their functional activity is different in cancer patients. Yokokawa et al. [102] showed that Tregs in patients with prostate carcinoma had an increased functionality compared with the healthy donors, which could be an important factor in the suppression of tumor-specific immune responses in these patients. Increased activity of Tregs can be caused by tumor- or stroma-derived immunosuppressive factors, such as PgE2, TGF-β and IL-10 [103, 104].

The prognostic significance of Treg infiltration was studied extensively and showed conflicting results. It has been associated with poor prognosis is some malignancies [97, 105–107] and no clinical significance in others [108–110]. It is also important to consider that Tregs could reduce the efficacy of immunotherapeutic protocols and thus, depletion of these cells could enhance vaccine-mediated anti-tumor immune responses and the efficacy of chemotherapy [111, 112].

Myeloid-derived suppressor cells

MDSCs are heterogeneous populations of immature myeloid cells accumulating in blood, lymph nodes, bone marrow and tumor sites in experimental animals and patients with cancer. They are capable of inhibiting both innate and adaptive immune responses [113] and their accumulation represents an important mechanism of tumor immune evasion [114, 115].

From the practical standpoint, the number of MDSCs is significantly higher in cancer patients compared with age- and sex-matched controls [116–120]. It is possible that some cancers are associated with profound immunosuppression even at an early stage while other cancers may only generate severe systemic immunosuppression when metastatic.

The frequency of each MDSCs subset appears to be influenced by cancer type. For some types of human cancers, such as renal cell carcinoma, glioma and bladder cancer, granulocytic MDSCs is the prevalent population in peripheral blood [121, 122], whereas, in patients with melanoma, multiple myeloma, prostate and hepatocellular carcinoma, monocytic MDSCs is the prominent population [123–125]. In addition, a population of MDSCs that express neither monocytic (CD14) nor granulocytic (CD15, CD16) markers and therefore cannot be categorized into one of the two main populations, has been demonstrated in the blood of patients with glioblastoma, breast cancer, colon cancer, lung cancer and kidney cancer [120, 126, 127]. The frequency and number of these cells has been shown to reflect the tumor burden, and a high frequency correlates with a poor prognosis and radiographic progression in a small number of patients with breast or colorectal cancer [119].

Similar to peripheral blood, elevated level of MDSCs are found in the tumor microenvironment of different cancer types compared with the surrounding non-cancerous tissues [116, 128, 129]. Recently, Sun et al. [116] reported that increased percentage of HLA-DR-CD33+ MDSCs in colorectal cancer correlated with tumor stage and distant metastasis. Gabitass et al. [118] found that density of intratumoral MDSCs was an independent prognostic factor in patients with pancreatic, esophageal and gastric carcinoma. The authors suggested that MDSC percentage could become a parameter for routine use in the prognostic modeling of these diseases.

Clinical evaluation of the immune system in cancer patients

Review of the studies presented above delineates several immunologic parameters that are ready to be included in a clinical evaluation of cancer patients. This list may not be comprehensive, but definitely includes such anti-tumor immune cells as NK cells, conventional DCs and cytotoxic T lymphocytes, along with pro-tumor immune cells, such as type 2 neutrophils, type 2 macrophages, plasmacytoid DCs, MDSCs and regulatory T lymphocytes. Each of these types of cells has been shown to influence clinical outcome; however, it is still unclear which ones have the highest importance or dominant significance. Thus, far, there are no studies that analyze all of these parameters in a large patient cohort both at the local level (in the tumor microenvironment) and at the systemic level (in peripheral blood, bone marrow, etc.). Nevertheless, remarkable advances in phenotypic and functional characterization of immune cells by immunohistochemical and flow cytometric methods have made it possible to perform a comprehensive evaluation of the immune status of cancer patients. The scheme of this evaluation will differ in patients with early stages and late stages of the disease and will significantly depend on the resectability of the primary tumor mass. We believe that in patients with resectable solid tumors such an evaluation may include five major steps.

Step one Malignancy is suspected based on a clinical presentation (pain, tumor mass, etc.)

Step two Tissue diagnosis is established by sampling the tumor mass (biopsy or cytologic evaluation). These tests usually characterize the type and grade of the tumor, but are insufficient for the evaluation of immune infiltrate in the tumor microenvironment.

Step three The patient's baseline immune status is evaluated. The tests include complete blood cell count with differentials and immunoglobulin concentrations and are routinely performed as part of the initial patient evaluation. These tests determine the status of the cellular and humoral immune systems and can detect immune deficiencies underlying the malignant process. In addition, based on the accumulating knowledge of the role of immunosuppressive types of cells (MDSCs, Tregs, etc.), flow cytometric analysis is performed to detect the initial levels of these cells in the peripheral blood or bone marrow (Table 2). Functional immunologic tests can be performed to assess immunosuppressive activity of these cells [130]. As was discussed above, the quantity and quality of immunosuppressive cells depends significantly on the stage of the malignant disease, determines the patient's prognosis, and predicts the response to treatment.

Step four In patients with resectable tumors, the primary tumor mass should be excised and undergo a complete pathologic evaluation. At this point all of the characteristics of the malignant tumor and of the tumor-infiltrating leukocytes can be determined.

Evaluation of tumor-infiltrating leukocytes can be performed by two major methodological approaches: microscopic examination of tumor sections (either fresh or fixed) and flow cytometric analysis of the fresh tumor tissue. Each of these methods has strength and weakness, and the best results can be achieved through a combination of both approaches. Microscopic analysis is performed by a qualified pathologist. Upon this analysis, pathologists notice the presence or absence of a specific type of tumor-infiltrating leukocytes in a tumor tissue. As discussed above, there is a

significant variation in the density of immune cells in different kinds of tumors. Quite often there is a predilection of a specific type of leukocytes to a specific type of cancer. Also, microscopic analysis can determine a spatial distribution of immune cells in the tumor mass. Tumor-infiltrating leukocytes can be located within cancer cell nests (intratumoral distribution), in the central cancer stroma (stromal distribution), and along the invasive tumor margins (peritumoral distribution). Since immune cells can have a dissimilar effect on malignant cells and stromal cells, the exact location of leukocytes is very important in the evaluation of their role. At the same time, the effect of leukocytes on the tumor depends on their functional status and the level of maturation. These parameters can also be tested for some of the cell types.

Next, density of tumor-infiltrating leukocytes can be correlated with the stage of a tumor. The process of tumor development, especially for epithelial tumors (carcinomas), includes steps such as cellular dysplasia, carcinoma in situ (non-invasive), locally invasive neoplasm and metastatic dissemination. Several studies describe the correlation between tumor-infiltrating leukocyte density and the tumor stage, which helps elucidate the involvement of immune cells in the tumor progression. Another correlation that is frequently performed during pathologic examination is that of leukocytes density with the tumor grade, proliferation index and HLA expression. Depending on the level of morphologic atypia, malignant tumors are classified as well differentiated, moderately differentiated, or poorly differentiated. Consistent correlations are found between tumor-infiltrating leukocytes density and tumor grade for many neoplasms. Finally, tumor-infiltrating leukocyte density can be correlated with the disease progression, clinical course, outcome and response to treatment.

From the technical standpoint, some of the tumor-infiltrating leukocytes, like neutrophils and lymphocytes, can be easily recognized by routine histochemical stains (e.g., H & E). However, these histochemical stains do not allow recognition of tumor-infiltrating leukocytes with certainty. In addition, they cannot discriminate between different subpopulations of cells or determine their state of maturation. Thus, evaluation of tumor-infiltrating leukocyte frequently requires utilization of methods that can determine not only morphology of the cells, but also their molecular phenotype. In pathology practice this is usually accomplished by immunohistochemistry that detects specific protein expression in the cells of interest (Table 1).

Immunohistochemistry can be performed on fresh or fixed tissue. Stained cells are counted under high magnification (usually, 400× or 1,000×), and the results are presented in a quantitative manner (e.g., number of cells per high power field) or in a semiquantitative manner (e.g., absent, weak, moderate, brisk infiltration). An excellent example of this approach is the study by Galon et al. of human colon cancer [45, 131, 132]. The authors proposed to classify colon tumors on the basis of an immune score for CD45RO memory T cells and cytotoxic CD8+ T cells in two tumor regions (central tumor and invasive margin). Using this immune score, five groups were defined (Im0, Im1, Im2, Im3, Im4). Patients with low densities of CD45RO and CD8 in both tumor regions were classified as Im0. Patients with one high density for one marker were classified as Im1. Patients with two, three, or four high densities among these markers were classified as Im2, Im3 and Im4, respectively. Statistical analysis showed a remarkable correlation of the immune score with clinical outcome; patients with Im4 had a 5-year disease-free survival of 85.4 %, whereas patients with Im0 had a 5-year disease-free survival of 31.6 %. These studies show that even a limited number of immunologic parameters can provide valuable diagnostic information. However, since different malignant tumors have different populations of tumor-infiltrating leukocytes, it is conceivable that other cell populations need to be included in the immune score. Specifically, the cells with pro-tumor and immunosuppressive effects (M2 macrophages, MDSCs, etc.) should be considered in the final analysis.

Although microscopic examination of tumor tissues is a powerful tool, it has several important limitations. First, it cannot evaluate the presence of tumor-infiltrating leukocytes for which there is no reliable histochemical or immunohistochemical marker. One such type of cells is MDSCs. Second, it cannot determine the functional state of tumor-infiltrating leukocytes. Both of these limitations can be overcome by flow cytometry analysis of fresh tumor tissue (Table 2). For example, in a recent study of Porembka et al., resected human pancreatic carcinoma specimens were analyzed by flow cytometry and showed a significant increase in MDSCs compared with normal pancreas tissue [133]. This approach is especially valuable in the analysis of tumor-infiltrating MDSCs, Treg cells, DCs and macrophages. However, flow cytometry has its own limitations. Preparation of single cell suspensions prevents determination of the spatial distribution of tumor-infiltrating leukocytes, and the need for a fresh tissue significantly limits the use of archival material.

In the majority of cases, there is a sufficient amount of tumor tissue for multiple immunohistochemical stains and flow cytometry. The limiting factor in a clinical setting is the availability of technical expertise and resources to perform these tests. At this time, there are no universally recommended test panels, specifically designed to analyze tumor-infiltrating leukocytes. This leaves the extent of testing to the discretion of a pathologist performing tissue examination. Ideally, the density and the spatial

distribution of both anti-tumor and pro-tumor immune cells should be evaluated.

In summary, when studying tumor-infiltrating leukocytes in a resected tumor, the following questions can be answered by a combination of microscopic and flow cytometric analyses:

1. Are these cells present in the tumor tissue and in what numbers?
2. What is their functional status and level of maturation?
3. What is the spatial distribution of leukocytes in correlation with tumor cell features (e.g., proliferation, apoptosis and necrosis) and stromal features (e.g., angiogenesis)?
4. Is there a correlation of density, state of maturation or spatial distribution of tumor-infiltrating leukocytes with tumor grade, stage and prognosis?

If the primary tumor can be successfully resected and there are no detectable metastases, the patient is considered "disease-free," although in the majority of cases, metastatic malignant cells would be present in lymph nodes and other body sites. This means that the clinical outcome of the disease depends on the interaction of these malignant cells with the factors of the metastatic microenvironment, particularly, the status of immune cells [134, 135]. This concept is based on numerous studies showing strong correlations between immune contexture of the primary tumor, immune status after surgical resection of tumors and patients' disease-free survival. We also have to consider that removal of the primary tumor may significantly influence the immune status of the patient. For example, research has shown that resection of colon cancer caused a significant decrease in circulating CD4+ CD25+ Foxp3+ Tregs [136]. Furthermore, after primary tumor resections, patients may receive adjuvant chemo- and radiotherapy. Chemotherapeutic agents have a substantial effect on different types of immune cells, altering their number and functionality and thus changing the overall immune profile of the patient [137, 138]. At the same time, patient's immune status, especially the number and activity of immunosuppressive cells, can influence the response to chemotherapy.

Therefore, there is a *step five* of evaluation: post-resection immunomonitoring. The tests that can be utilized are similar to those used in step three and include complete blood cell count with differentials, immunoglobulin concentrations, flow cytometry analysis of immune cell populations and functional immunologic studies. Commonly used functional assays (e.g., IFN-γ ELISPOT assay) have recently been comprehensively reviewed [130]. The results of these tests have high prognostic and predictive value and help stratify patients to high- and low-risk groups, which makes them a valuable tool for making adjuvant therapy decisions.

Conclusion

Significant advances in experimental and clinical oncoimmunology achieved in the last decade have opened an opportunity for the use of modern morphologic, flow cytometric and functional tests in everyday clinical practice. Although inclusion of these test in routine evaluation of cancer patients comes with a significant increase in workload and expense, their prognostic and predictive value definitely justifies their use. Stratification of patients according to their immune status in the course of the disease will help to identify high-risk patient populations that need close follow-up and aggressive treatment. Identification of the most prevalent and functionally active immune cell populations (i.e., MDSCs, Treg cells, M1/M2 macrophages, etc.) both at the local and systemic levels can lead to the use of novel specific types of personalized immunotherapy. There is no consensus yet regarding the types of immune cells that need to be evaluated and the modes of laboratory tests most appropriate for this process, but the extensive ongoing clinical work suggests that a major breakthrough in the field of tumor immunology is forthcoming.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Smyth MJ, Dunn GP, Schreiber RD (2006) Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. Adv Immunol 90:1–50
2. Papamichail M, Perez SA, Gritzapis AD, Baxevanis CN (2004) Natural killer lymphocytes: biology, development, and function. Cancer Immunol Immunother 53(3):176–186
3. Orange JS, Ballas ZK (2006) Natural killer cells in human health and disease. Clin Immunol 118(1):1–10
4. Villegas FR, Coca S, Villarrubia VG, Jimenez R, Chillon MJ, Jareno J, Zuil M, Callol L (2002) Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. Lung Cancer (Amsterdam, Netherlands) 35(1):23–28
5. Ishigami S, Natsugoe S, Tokuda K, Nakajo A, Che X, Iwashige H, Aridome K, Hokita S, Aikou T (2000) Prognostic value of intratumoral natural killer cells in gastric carcinoma. Cancer 88(3):577–583
6. Coca S, Perez-Piqueras J, Martinez D, Colmenarejo A, Saez MA, Vallejo C, Martos JA, Moreno M (1997) The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. Cancer 79(12):2320–2328
7. Schleypen JS, Baur N, Kammerer R, Nelson PJ, Rohrmann K, Grone EF, Hohenfellner M, Haferkamp A, Pohla H, Schendel DJ, Falk CS, Noessner E (2006) Cytotoxic markers and frequency predict functional capacity of natural killer cells infiltrating renal cell carcinoma. Clin Cancer Res 12(3 Pt 1):718–725
8. Al-Shibli K, Al-Saad S, Donnem T, Persson M, Bremnes RM, Busund LT (2009) The prognostic value of intraepithelial and

stromal innate immune system cells in non-small cell lung carcinoma. Histopathology 55(3):301–312
9. Sorbye SW, Kilvaer TK, Valkov A, Donnem T, Smeland E, Al-Shibli K, Bremnes RM, Busund LT (2012) Prognostic impact of CD57, CD68, M-CSF, CSF-1R, Ki67 and TGF-beta in soft tissue sarcomas. BMC Clin Pathol 12:7
10. Bell D, Chomarat P, Broyles D, Netto G, Harb GM, Lebecque S, Valladeau J, Davoust J, Palucka KA, Banchereau J (1999) In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas. J Exp Med 190(10):1417–1426
11. Colasante A, Castrilli G, Aiello FB, Brunetti M, Musiani P (1995) Role of cytokines in distribution and differentiation of dendritic cell/Langerhans' cell lineage in human primary carcinomas of the lung. Hum Pathol 26(8):866–872
12. Inoshima N, Nakanishi Y, Minami T, Izumi M, Takayama K, Yoshino I, Hara N (2002) The influence of dendritic cell infiltration and vascular endothelial growth factor expression on the prognosis of non-small cell lung cancer. Clin Cancer Res 8(11):3480–3486
13. Zeid NA, Muller HK (1993) S100 positive dendritic cells in human lung tumors associated with cell differentiation and enhanced survival. Pathology 25(4):338–343
14. Katsenelson NS, Shurin GV, Bykovskaia SN, Shogan J, Shurin MR (2001) Human small cell lung carcinoma and carcinoid tumor regulate dendritic cell maturation and function. Mod Pathol 14(1):40–45
15. Inoue K, Furihata M, Ohtsuki Y, Fujita Y (1993) Distribution of S-100 protein-positive dendritic cells and expression of HLA-DR antigen in transitional cell carcinoma of the urinary bladder in relation to tumour progression and prognosis. Virchows Arch 422(5):351–355
16. Reichert TE, Scheuer C, Day R, Wagner W, Whiteside TL (2001) The number of intratumoral dendritic cells and zeta-chain expression in T cells as prognostic and survival biomarkers in patients with oral carcinoma. Cancer 91(11): 2136–2147
17. Dallal RM, Christakos P, Lee K, Egawa S, Son YI, Lotze MT (2002) Paucity of dendritic cells in pancreatic cancer. Surgery 131(2):135–138
18. Troy A, Davidson P, Atkinson C, Hart D (1998) Phenotypic characterisation of the dendritic cell infiltrate in prostate cancer. J Urol 160(1):214–219
19. Tsukayama S, Omura K, Yoshida K, Tanaka Y, Watanabe G (2005) Prognostic value of CD83-positive mature dendritic cells and their relation to vascular endothelial growth factor in advanced human gastric cancer. Oncol Rep 14(2):369–375
20. Vakkila J, Jaffe R, Michelow M, Lotze MT (2006) Pediatric cancers are infiltrated predominantly by macrophages and contain a paucity of dendritic cells: a major nosologic difference with adult tumors. Clin Cancer Res 12(7 Pt 1):2049–2054
21. Nagorsen D, Voigt S, Berg E, Stein H, Thiel E, Loddenkemper C (2007) Tumor-infiltrating macrophages and dendritic cells in human colorectal cancer: relation to local regulatory T cells, systemic T-cell response against tumor-associated antigens and survival. J Transl Med 5:62
22. Kurabayashi A, Furihata M, Matsumoto M, Hayashi H, Ohtsuki Y (2004) Distribution of tumor-infiltrating dendritic cells in human non-small cell lung carcinoma in relation to apoptosis. Pathol Int 54(5):302–310
23. Lespagnard L, Gancberg D, Rouas G, Leclercq G, de Saint-Aubain Somerhausen N, Di Leo A, Piccart M, Verhest A, Larsimont D (1999) Tumor-infiltrating dendritic cells in adenocarcinomas of the breast: a study of 143 neoplasms with a correlation to usual prognostic factors and to clinical outcome. Int J Cancer 84(3):309–314
24. Coventry BJ, Lee PL, Gibbs D, Hart DN (2002) Dendritic cell density and activation status in human breast cancer—CD1a, CMRF-44, CMRF-56 and CD-83 expression. Br J Cancer 86(4):546–551
25. Miyagawa S, Soeda J, Takagi S, Miwa S, Ichikawa E, Noike T (2004) Prognostic significance of mature dendritic cells and factors associated with their accumulation in metastatic liver tumors from colorectal cancer. Hum Pathol 35(11):1392–1396
26. O'Donnell RK, Mick R, Feldman M, Hino S, Wang Y, Brose MS, Muschel RJ (2007) Distribution of dendritic cell subtypes in primary oral squamous cell carcinoma is inconsistent with a functional response. Cancer Lett 255(1):145–152
27. Furihata M, Ono Y, Ichikawa K, Tomita S, Fujimori T, Kubota K (2005) Prognostic significance of CD83 positive, mature dendritic cells in the gallbladder carcinoma. Oncol Rep 14(2):353–356
28. Troy AJ, Davidson PJ, Atkinson CH, Hart DN (1999) CD1a dendritic cells predominate in transitional cell carcinoma of bladder and kidney but are minimally activated. J Urol 161(6):1962–1967
29. Vermi W, Bonecchi R, Facchetti F, Bianchi D, Sozzani S, Festa S, Berenzi A, Cella M, Colonna M (2003) Recruitment of immature plasmacytoid dendritic cells (plasmacytoid monocytes) and myeloid dendritic cells in primary cutaneous melanomas. J Pathol 200(2):255–268
30. Bigotti G, Coli A, Castagnola D (1991) Distribution of Langerhans cells and HLA class II molecules in prostatic carcinomas of different histopathological grade. Prostate 19(1):73–87
31. Coppola D, Fu L, Nicosia SV, Kounelis S, Jones M (1998) Prognostic significance of p53, bcl-2, vimentin, and S100 protein-positive Langerhans cells in endometrial carcinoma. Hum Pathol 29(5):455–462
32. Kikuchi K, Kusama K, Taguchi K, Ishikawa F, Okamoto M, Shimada J, Sakashita H, Yamamo Y (2002) Dendritic cells in human squamous cell carcinoma of the oral cavity. Anticancer Res 22(2A):545–557
33. Hayati AR, Zulkarnaen M (2007) An immunohistochemical study of CD1a and CD83-positive infiltrating dendritic cell density in cervical neoplasia. Int J Gynecol Pathol 26(1):83–88
34. Dieu-Nosjean MC, Antoine M, Danel C, Heudes D, Wislez M, Poulot V, Rabbe N, Laurans L, Tartour E, de Chaisemartin L, Lebecque S, Fridman WH, Cadranel J (2008) Long-term survival for patients with non-small-cell lung cancer with intratumoral lymphoid structures. J Clin Oncol 26(27):4410–4417
35. Dai F, Liu L, Che G, Yu N, Pu Q, Zhang S, Ma J, Ma L, You Z (2010) The number and microlocalization of tumor-associated immune cells are associated with patient's survival time in non-small cell lung cancer. BMC Cancer 10:220
36. Treilleux I, Blay JY, Bendriss-Vermare N, Ray-Coquard I, Bachelot T, Guastalla JP, Bremond A, Goddard S, Pin JJ, Barthelemy-Dubois C, Lebecque S (2004) Dendritic cell infiltration and prognosis of early stage breast cancer. Clin Cancer Res 10(22):7466–7474
37. Coventry BJ, Morton J (2003) CD1a-positive infiltrating-dendritic cell density and 5-year survival from human breast cancer. Br J Cancer 89(3):533–538
38. Cai XY, Gao Q, Qiu SJ, Ye SL, Wu ZQ, Fan J, Tang ZY (2006) Dendritic cell infiltration and prognosis of human hepatocellular carcinoma. J Cancer Res Clin Oncol 132(5):293–301
39. Simonetti O, Goteri G, Lucarini G, Rubini C, Stramazzotti D, Lo Muzio L, Biagini G, Offidani A (2007) In melanoma changes of immature and mature dendritic cell expression correlate with tumor thickness: an immunohistochemical study. Int J Immunopathol Pharmacol 20(2):325–333
40. Al-Shibli K, Al-Saad S, Andersen S, Donnem T, Bremnes RM, Busund LT (2010) The prognostic value of intraepithelial and

stromal CD3-, CD117- and CD138-positive cells in non-small cell lung carcinoma. Apmis 118(5):371–382
41. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoue F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pages F (2006) Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 313(5795):1960–1964
42. Lee CH, Espinosa I, Vrijaldenhoven S, Subramanian S, Montgomery KD, Zhu S, Marinelli RJ, Peterse JL, Poulin N, Nielsen TO, West RB, Gilks CB, van de Rijn M (2008) Prognostic significance of macrophage infiltration in leiomyosarcomas. Clin Cancer Res 14(5):1423–1430
43. Tomsova M, Melichar B, Sedlakova I, Steiner I (2008) Prognostic significance of CD3+ tumor-infiltrating lymphocytes in ovarian carcinoma. Gynecol Oncol 108(2):415–420
44. Gooden MJ, de Bock GH, Leffers N, Daemen T, Nijman HW (2011) The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. Br J Cancer 105(1):93–103
45. Mlecnik B, Tosolini M, Kirilovsky A, Berger A, Bindea G, Meatchi T, Bruneval P, Trajanoski Z, Fridman WH, Pages F, Galon J (2011) Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. J Clin Oncol 29(6):610–618
46. Schumacher K, Haensch W, Roefzaad C, Schlag PM (2001) Prognostic significance of activated CD8(+) T cell infiltrations within esophageal carcinomas. Cancer Res 61(10): 3932–3936
47. Sharma P, Shen Y, Wen S, Yamada S, Jungbluth AA, Gnjatic S, Bajorin DF, Reuter VE, Herr H, Old LJ, Sato E (2007) CD8 tumor-infiltrating lymphocytes are predictive of survival in muscle-invasive urothelial carcinoma. Proc Natl Acad Sci USA 104(10):3967–3972
48. Oshikiri T, Miyamoto M, Shichinohe T, Suzuoki M, Hiraoka K, Nakakubo Y, Shinohara T, Itoh T, Kondo S, Katoh H (2003) Prognostic value of intratumoral CD8+ T lymphocyte in extrahepatic bile duct carcinoma as essential immune response. J Surg Oncol 84(4):224–228
49. Kondratiev S, Sabo E, Yakirevich E, Lavie O, Resnick MB (2004) Intratumoral CD8+ T lymphocytes as a prognostic factor of survival in endometrial carcinoma. Clin Cancer Res 10(13):4450–4456
50. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, Jungbluth AA, Frosina D, Gnjatic S, Ambrosone C, Kepner J, Odunsi T, Ritter G, Lele S, Chen YT, Ohtani H, Old LJ, Odunsi K (2005) Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci USA 102(51):18538–18543
51. Wakabayashi O, Yamazaki K, Oizumi S, Hommura F, Kinoshita I, Ogura S, Dosaka-Akita H, Nishimura M (2003) CD4+ T cells in cancer stroma, not CD8+ T cells in cancer cell nests, are associated with favorable prognosis in human non-small cell lung cancers. Cancer Sci 94(11):1003–1009
52. Ishibashi S, Ohashi Y, Suzuki T, Miyazaki S, Moriya T, Satomi S, Sasano H (2006) Tumor-associated tissue eosinophilia in human esophageal squamous cell carcinoma. Anticancer Res 26(2B):1419–1424
53. Sorbye SW, Kilvaer T, Valkov A, Donnem T, Smeland E, Al-Shibli K, Bremnes RM, Busund LT (2011) Prognostic impact of lymphocytes in soft tissue sarcomas. PLoS ONE 6(1):e14611
54. Al-Shibli KI, Donnem T, Al-Saad S, Persson M, Bremnes RM, Busund LT (2008) Prognostic effect of epithelial and stromal lymphocyte infiltration in non-small cell lung cancer. Clin Cancer Res 14(16):5220–5227
55. Pelletier MP, Edwardes MD, Michel RP, Halwani F, Morin JE (2001) Prognostic markers in resectable non-small cell lung cancer: a multivariate analysis. Can J Surg 44(3):180–188
56. Sorbye SW, Kilvaer T, Valkov A, Donnem T, Smeland E, Al-Shibli K, Bremnes RM, Busund LT (2012) High expression of CD20+ lymphocytes in soft tissue sarcomas is a positive prognostic indicator. Oncoimmunology 1(1):75–77
57. Hiraoka K, Miyamoto M, Cho Y, Suzuoki M, Oshikiri T, Nakakubo Y, Itoh T, Ohbuchi T, Kondo S, Katoh H (2006) Concurrent infiltration by CD8+ T cells and CD4+ T cells is a favourable prognostic factor in non-small-cell lung carcinoma. Br J Cancer 94(2):275–280
58. Cho Y, Miyamoto M, Kato K, Fukunaga A, Shichinohe T, Kawarada Y, Hida Y, Oshikiri T, Kurokawa T, Suzuoki M, Nakakubo Y, Hiraoka K, Murakami S, Shinohara T, Itoh T, Okushiba S, Kondo S, Katoh H (2003) CD4+ and CD8+ T cells cooperate to improve prognosis of patients with esophageal squamous cell carcinoma. Cancer Res 63(7):1555–1559
59. Rice AJ, Griffiths AP, Martin IG, Dixon MF (2000) Gastric carcinoma with prominent neutrophil infiltration. Histopathology 37(3):289–290
60. Jensen HK, Donskov F, Marcussen N, Nordsmark M, Lundbeck F, von der Maase H (2009) Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma. J Clin Oncol 27(28):4709–4717
61. Wislez M, Rabbe N, Marchal J, Milleron B, Crestani B, Mayaud C, Antoine M, Soler P, Cadranel J (2003) Hepatocyte growth factor production by neutrophils infiltrating bronchioloalveolar subtype pulmonary adenocarcinoma: role in tumor progression and death. Cancer Res 63(6):1405–1412
62. Bellocq A, Antoine M, Flahault A, Philippe C, Crestani B, Bernaudin JF, Mayaud C, Milleron B, Baud L, Cadranel J (1998) Neutrophil alveolitis in bronchioloalveolar carcinoma: induction by tumor-derived interleukin-8 and relation to clinical outcome. Am J Pathol 152(1):83–92
63. Tazzyman S, Lewis CE, Murdoch C (2009) Neutrophils: key mediators of tumour angiogenesis. Int J Exp Pathol 90(3): 222–231
64. Coffelt SB, Lewis CE, Naldini L, Brown JM, Ferrara N, De Palma M (2010) Elusive identities and overlapping phenotypes of proangiogenic myeloid cells in tumors. Am J Pathol 176(4):1564–1576
65. Gregory AD, Houghton AM (2011) Tumor-associated neutrophils: new targets for cancer therapy. Cancer Res 71(7):2411–2416
66. Zhao JJ, Pan K, Wang W, Chen JG, Wu YH, Lv L, Li JJ, Chen YB, Wang DD, Pan QZ, Li XD, Xia JC (2012) The prognostic value of tumor-infiltrating neutrophils in gastric adenocarcinoma after resection. PLoS ONE 7(3):e33655
67. Rao HL, Chen JW, Li M, Xiao YB, Fu J, Zeng YX, Cai MY, Xie D (2012) Increased intratumoral neutrophil in colorectal carcinomas correlates closely with malignant phenotype and predicts patients' adverse prognosis. PLoS ONE 7(1):e30806
68. Nielsen HJ, Hansen U, Christensen IJ, Reimert CM, Brunner N, Moesgaard F (1999) Independent prognostic value of eosinophil and mast cell infiltration in colorectal cancer tissue. J Pathol 189(4):487–495
69. Caruso RA, Bellocco R, Pagano M, Bertoli G, Rigoli L, Inferrera C (2002) Prognostic value of intratumoral neutrophils in advanced gastric carcinoma in a high-risk area in northern Italy. Mod Pathol 15(8):831–837
70. Sickert D, Aust DE, Langer S, Haupt I, Baretton GB, Dieter P (2005) Characterization of macrophage subpopulations in colon cancer using tissue microarrays. Histopathology 46(5):515–521
71. Talmadge JE, Donkor M, Scholar E (2007) Inflammatory cell infiltration of tumors: Jekyll or Hyde. Cancer Metastasis Rev 26(3–4):373–400

72. Mantovani A, Sica A, Locati M (2007) New vistas on macrophage differentiation and activation. Eur J Immunol 37(1):14–16
73. Solinas G, Germano G, Mantovani A, Allavena P (2009) Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. J Leukoc Biol 86(5):1065–1073
74. Lewis CE, Pollard JW (2006) Distinct role of macrophages in different tumor microenvironments. Cancer Res 66(2):605–612
75. Mosser DM, Edwards JP (2008) Exploring the full spectrum of macrophage activation. Nat Rev Immunol 8(12):958–969
76. Pollard JW (2004) Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 4(1):71–78
77. Ohtaki Y, Ishii G, Nagai K, Ashimine S, Kuwata T, Hishida T, Nishimura M, Yoshida J, Takeyoshi I, Ochiai A (2010) Stromal macrophage expressing CD204 is associated with tumor aggressiveness in lung adenocarcinoma. J Thorac Oncol 5(10):1507–1515
78. Kurahara H, Shinchi H, Mataki Y, Maemura K, Noma H, Kubo F, Sakoda M, Ueno S, Natsugoe S, Takao S (2009) Significance of M2-polarized tumor-associated macrophage in pancreatic cancer. J Surg Res 167(2):e211–e219
79. Komohara Y, Hasita H, Ohnishi K, Fujiwara Y, Suzu S, Eto M, Takeya M (2011) Macrophage infiltration and its prognostic relevance in clear cell renal cell carcinoma. Cancer Sci 102(7):1424–1431
80. Espinosa I, Jose Carnicer M, Catasus L, Canet B, D'Angelo E, Zannoni GF, Prat J (2010) Myometrial invasion and lymph node metastasis in endometrioid carcinomas: tumor-associated macrophages, microvessel density, and HIF1A have a crucial role. Am J Surg Pathol 34(11):1708–1714
81. Matta BM, Castellaneta A, Thomson AW (2010) Tolerogenic plasmacytoid DC. Eur J Immunol 40(10):2667–2676
82. Vermi W, Soncini M, Melocchi L, Sozzani S, Facchetti F (2011) Plasmacytoid dendritic cells and cancer. J Leukoc Biol 90(4):681–690
83. Labidi-Galy SI, Sisirak V, Meeus P, Gobert M, Treilleux I, Bajard A, Combes JD, Faget J, Mithieux F, Cassignol A, Tredan O, Durand I, Menetrier-Caux C, Caux C, Blay JY, Ray-Coquard I, Bendriss-Vermare N (2011) Quantitative and functional alterations of plasmacytoid dendritic cells contribute to immune tolerance in ovarian cancer. Cancer Res 71(16):5423–5434
84. Thiel A, Pries R, Jeske S, Trenkle T, Wollenberg B (2009) Effect of head and neck cancer supernatant and CpG-oligonucleotides on migration and IFN-alpha production of plasmacytoid dendritic cells. Anticancer Res 29(8):3019–3025
85. Watkins SK, Zhu Z, Riboldi E, Shafer-Weaver KA, Stagliano KE, Sklavos MM, Ambs S, Yagita H, Hurwitz AA (2011) FOXO3 programs tumor-associated DCs to become tolerogenic in human and murine prostate cancer. J Clin Investig 121(4): 1361–1372
86. Norian LA, Rodriguez PC, O'Mara LA, Zabaleta J, Ochoa AC, Cella M, Allen PM (2009) Tumor-infiltrating regulatory dendritic cells inhibit CD8+ T cell function via L-arginine metabolism. Cancer Res 69(7):3086–3094
87. Jahrsdorfer B, Vollmer A, Blackwell SE, Maier J, Sontheimer K, Beyer T, Mandel B, Lunov O, Tron K, Nienhaus GU, Simmet T, Debatin KM, Weiner GJ, Fabricius D (2010) Granzyme B produced by human plasmacytoid dendritic cells suppresses T-cell expansion. Blood 115(6):1156–1165
88. Sharma MD, Baban B, Chandler P, Hou DY, Singh N, Yagita H, Azuma M, Blazar BR, Mellor AL, Munn DH (2007) Plasmacytoid dendritic cells from mouse tumor-draining lymph nodes directly activate mature Tregs via indoleamine 2,3-dioxygenase. J Clin Investig 117(9):2570–2582
89. Woo EY, Chu CS, Goletz TJ, Schlienger K, Yeh H, Coukos G, Rubin SC, Kaiser LR, June CH (2001) Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. Cancer Res 61(12):4766–4772
90. Tokuno K, Hazama S, Yoshino S, Yoshida S, Oka M (2009) Increased prevalence of regulatory T-cells in the peripheral blood of patients with gastrointestinal cancer. Anticancer Res 29(5):1527–1532
91. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 10(9):942–949
92. Schaefer C, Kim GG, Albers A, Hoermann K, Myers EN, Whiteside TL (2005) Characteristics of CD4+ CD25+ regulatory T cells in the peripheral circulation of patients with head and neck cancer. Br J Cancer 92(5):913–920
93. Mathai AM, Kapadia MJ, Alexander J, Kernochan LE, Swanson PE, Yeh MM (2012) Role of Foxp3-positive tumor-infiltrating lymphocytes in the histologic features and clinical outcomes of hepatocellular carcinoma. Am J Surg Pathol 36(7):980–986
94. Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, Drebin JA, Strasberg SM, Eberlein TJ, Goedegebuure PS, Linehan DC (2002) Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. J Immunol 169(5):2756–2761
95. Miller AM, Lundberg K, Ozenci V, Banham AH, Hellstrom M, Egevad L, Pisa P (2006) CD4+ CD25high T cells are enriched in the tumor and peripheral blood of prostate cancer patients. J Immunol 177(10):7398–7405
96. Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H, Fujii H (2003) Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. Clin Cancer Res 9(12):4404–4408
97. Bates GJ, Fox SB, Han C, Leek RD, Garcia JF, Harris AL, Banham AH (2006) Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. J Clin Oncol 24(34):5373–5380
98. Gao Q, Qiu SJ, Fan J, Zhou J, Wang XY, Xiao YS, Xu Y, Li YW, Tang ZY (2007) Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. J Clin Oncol 25(18):2586–2593
99. Ishibashi Y, Tanaka S, Tajima K, Yoshida T, Kuwano H (2006) Expression of Foxp3 in non-small cell lung cancer patients is significantly higher in tumor tissues than in normal tissues, especially in tumors smaller than 30 mm. Oncol Rep 15(5):1315–1319
100. De Panfilis G, Campanini N, Santini M, Mori G, Tognetti E, Maestri R, Lombardi M, Froio E, Ferrari D, Ricci R (2008) Phase- and stage-related proportions of T cells bearing the transcription factor FOXP3 infiltrate primary melanoma. J Invest Dermatol 128(3):676–684
101. Miracco C, Mourmouras V, Biagioli M, Rubegni P, Mannucci S, Monciatti I, Cosci E, Tosi P, Luzi P (2007) Utility of tumour-infiltrating CD25+ FOXP3+ regulatory T cell evaluation in predicting local recurrence in vertical growth phase cutaneous melanoma. Oncol Rep 18(5):1115–1122
102. Yokokawa J, Cereda V, Remondo C, Gulley JL, Arlen PM, Schlom J, Tsang KY (2008) Enhanced functionality of CD4+ CD25(high)FoxP3+ regulatory T cells in the peripheral blood of patients with prostate cancer. Clin Cancer Res 14(4):1032–1040
103. Viguier M, Lemaitre F, Verola O, Cho MS, Gorochov G, Dubertret L, Bachelez H, Kourilsky P, Ferradini L (2004) Foxp3 expressing CD4+ CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and

inhibit the function of infiltrating T cells. J Immunol 173(2):1444–1453
104. Liyanage UK, Goedegebuure PS, Moore TT, Viehl CT, Moo-Young TA, Larson JW, Frey DM, Ehlers JP, Eberlein TJ, Linehan DC (2006) Increased prevalence of regulatory T cells (Treg) is induced by pancreas adenocarcinoma. J Immunother 29(4):416–424
105. Shimizu K, Nakata M, Hirami Y, Yukawa T, Maeda A, Tanemoto K (2010) Tumor-infiltrating Foxp3+ regulatory T cells are correlated with cyclooxygenase-2 expression and are associated with recurrence in resected non-small cell lung cancer. J Thorac Oncol 5(5):585–590
106. Li JF, Chu YW, Wang GM, Zhu TY, Rong RM, Hou J, Xu M (2009) The prognostic value of peritumoral regulatory T cells and its correlation with intratumoral cyclooxygenase-2 expression in clear cell renal cell carcinoma. BJU Int 103(3):399–405
107. Shen Z, Zhou S, Wang Y, Li RL, Zhong C, Liang C, Sun Y (2010) Higher intratumoral infiltrated Foxp3+ Treg numbers and Foxp3+/CD8+ ratio are associated with adverse prognosis in resectable gastric cancer. J Cancer Res Clin Oncol 136(10): 1585–1595
108. Nosho K, Baba Y, Tanaka N, Shima K, Hayashi M, Meyerhardt JA, Giovannucci E, Dranoff G, Fuchs CS, Ogino S (2010) Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. J Pathol 222(4):350–366
109. Ladanyi A, Mohos A, Somlai B, Liszkay G, Gilde K, Fejos Z, Gaudi I, Timar J (2010) FOXP3+ cell density in primary tumor has no prognostic impact in patients with cutaneous malignant melanoma. Pathol Oncol Res 16(3):303–309
110. Suzuki H, Chikazawa N, Tasaka T, Wada J, Yamasaki A, Kitaura Y, Sozaki M, Tanaka M, Onishi H, Morisaki T, Katano M (2010) Intratumoral CD8(+) T/FOXP3 (+) cell ratio is a predictive marker for survival in patients with colorectal cancer. Cancer Immunol Immunother 59(5):653–661
111. Dannull J, Su Z, Rizzieri D, Yang BK, Coleman D, Yancey D, Zhang A, Dahm P, Chao N, Gilboa E, Vieweg J (2005) Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. J Clin Investig 115(12):3623–3633
112. Elkord E, Alcantar-Orozco EM, Dovedi SJ, Tran DQ, Hawkins RE, Gilham DE (2010) T regulatory cells in cancer: recent advances and therapeutic potential. Exp Opinion Biol Therapy 10(11):1573–1586
113. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V (2012) Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol 12(4):253–268
114. Fujimura T, Mahnke K, Enk AH (2010) Myeloid derived suppressor cells and their role in tolerance induction in cancer. J Dermatol Sci 59(1):1–6
115. Ostrand-Rosenberg S (2010) Myeloid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity. Cancer Immunol Immunother 59(10):1593–1600
116. Sun HL, Zhou X, Xue YF, Wang K, Shen YF, Mao JJ, Guo HF, Miao ZN (2012) Increased frequency and clinical significance of myeloid-derived suppressor cells in human colorectal carcinoma. World J Gastroenterol 18(25):3303–3309
117. Verschoor CP, Johnstone J, Millar J, Dorrington MG, Habibagahi M, Lelic A, Loeb M, Bramson JL, Bowdish DM (2013) Blood CD33(+)HLA-DR(-) myeloid-derived suppressor cells are increased with age and a history of cancer. J Leukoc Biol 93(4):633–637
118. Gabitass RF, Annels NE, Stocken DD, Pandha HA, Middleton GW (2011) Elevated myeloid-derived suppressor cells in pancreatic, esophageal and gastric cancer are an independent prognostic factor and are associated with significant elevation of the Th2 cytokine interleukin-13. Cancer Immunol Immunother 60(10):1419–1430
119. Diaz-Montero CM, Salem ML, Nishimura MI, Garrett-Mayer E, Cole DJ, Montero AJ (2009) Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. Cancer Immunol Immunother 58(1):49–59
120. Solito S, Falisi E, Diaz-Montero CM, Doni A, Pinton L, Rosato A, Francescato S, Basso G, Zanovello P, Onicescu G, Garrett-Mayer E, Montero AJ, Bronte V, Mandruzzato S (2011) A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells. Blood 118(8):2254–2265
121. Rodriguez PC, Ernstoff MS, Hernandez C, Atkins M, Zabaleta J, Sierra R, Ochoa AC (2009) Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. Cancer Res 69(4):1553–1560
122. Ko JS, Zea AH, Rini BI, Ireland JL, Elson P, Cohen P, Golshayan A, Rayman PA, Wood L, Garcia J, Dreicer R, Bukowski R, Finke JH (2009) Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients. Clin Cancer Res 15(6):2148–2157
123. Filipazzi P, Huber V, Rivoltini L (2012) Phenotype, function and clinical implications of myeloid-derived suppressor cells in cancer patients. Cancer Immunol Immunother 61(2):255–263
124. Vuk-Pavlovic S, Bulur PA, Lin Y, Qin R, Szumlanski CL, Zhao X, Dietz AB (2010) Immunosuppressive CD14+ HLA-DRlow/- monocytes in prostate cancer. Prostate 70(4):443–455
125. Najjar YG, Finke JH (2013) Clinical perspectives on targeting of myeloid derived suppressor cells in the treatment of cancer. Frontiers Oncol 3:49
126. Montero AJ, Diaz-Montero CM, Deutsch YE, Hurley J, Koniaris LG, Rumboldt T, Yasir S, Jorda M, Garret-Mayer E, Avisar E, Slingerland J, Silva O, Welsh C, Schuhwerk K, Seo P, Pegram MD, Gluck S (2012) Phase 2 study of neoadjuvant treatment with NOV-002 in combination with doxorubicin and cyclophosphamide followed by docetaxel in patients with HER-2 negative clinical stage II–IIIc breast cancer. Breast Cancer Res Treat 132(1):215–223
127. Raychaudhuri B, Rayman P, Ireland J, Ko J, Rini B, Borden EC, Garcia J, Vogelbaum MA, Finke J (2011) Myeloid-derived suppressor cell accumulation and function in patients with newly diagnosed glioblastoma. Neuro-oncology 13(6):591–599
128. Feng PH, Lee KY, Chang YL, Chan YF, Kuo LW, Lin TY, Chung FT, Kuo CS, Yu CT, Lin SM, Wang CH, Chou CL, Huang CD, Kuo HP (2012) CD14(+)S100A9(+) monocytic myeloid-derived suppressor cells and their clinical relevance in non-small cell lung cancer. Am J Respir Crit Care Med 186(10):1025–1036
129. Zhang B, Wang Z, Wu L, Zhang M, Li W, Ding J, Zhu J, Wei H, Zhao K (2013) Circulating and tumor-infiltrating myeloid-derived suppressor cells in patients with colorectal carcinoma. PLoS ONE 8(2):e57114
130. Malyguine AM, Strobl SL, Shurin MR (2012) Immunological monitoring of the tumor immunoenvironment for clinical trials. Cancer Immunol Immunother 61(2):239–247
131. Galon J, Pages F, Marincola FM, Angell HK, Thurin M, Lugli A, Zlobec I, Berger A, Bifulco C, Botti G, Tatangelo F, Britten CM, Kreiter S, Chouchane L, Delrio P, Arndt H, Asslaber M, Maio M, Masucci GV, Mihm M, Vidal-Vanaclocha F, Allison JP, Gnjatic S, Hakansson L, Huber C, Singh-Jasuja H, Ottensmeier C, Zwierzina H, Laghi L, Grizzi F, Ohashi PS, Shaw PA, Clarke BA, Wouters BG, Kawakami Y, Hazama S, Okuno K, Wang E, O'Donnell-Tormey J, Lagorce C, Pawelec G, Nishimura MI, Hawkins R, Lapointe R, Lundqvist A, Khleif SN, Ogino S, Gibbs P, Waring P, Sato N, Torigoe T, Itoh K, Patel PS, Shukla SN, Palmqvist R, Nagtegaal ID, Wang Y, D'Arrigo C, Kopetz S, Sinicrope FA, Trinchieri G, Gajewski TF, Ascierto

PA, Fox BA (2012) Cancer classification using the immunoscore: a worldwide task force. J Transl Med 10:205
132. Fridman WH, Galon J, Dieu-Nosjean MC, Cremer I, Fisson S, Damotte D, Pages F, Tartour E, Sautes-Fridman C (2011) Immune infiltration in human cancer: prognostic significance and disease control. Curr Top Microbiol Immunol 344:1–24
133. Porembka MR, Mitchem JB, Belt BA, Hsieh CS, Lee HM, Herndon J, Gillanders WE, Linehan DC, Goedegebuure P (2012) Pancreatic adenocarcinoma induces bone marrow mobilization of myeloid-derived suppressor cells which promote primary tumor growth. Cancer Immunol Immunother 61(9):1373–1385
134. Paez D, Labonte MJ, Bohanes P, Zhang W, Benhanim L, Ning Y, Wakatsuki T, Loupakis F, Lenz HJ (2012) Cancer dormancy: a model of early dissemination and late cancer recurrence. Clin Cancer Res 18(3):645–653
135. Spano D, Zollo M (2012) Tumor microenvironment: a main actor in the metastasis process. Clin Exp Metastasis 29(4): 381–395
136. Sellitto A, Galizia G, De Fanis U, Lieto E, Zamboli A, Orditura M, De Vita F, Giunta R, Lucivero G, Romano C (2011) Behavior of circulating CD4+ CD25+ Foxp3+ regulatory T cells in colon cancer patients undergoing surgery. J Clin Immunol 31(6):1095–1104
137. Naiditch H, Shurin MR, Shurin GV (2011) Targeting myeloid regulatory cells in cancer by chemotherapeutic agents. Immunol Res 50(2–3):276–285
138. Shurin MR (2013) Dual role of immunomodulation by anticancer chemotherapy. Nat Med 19(1):20–22